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# Molecular Phylogeny and Taxonomy of the Genus *Disporum* (Colchicaceae)

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In an effort to better understand phylogenetic relationships within *Disporum* (Colchicaceae), we conducted molecular analyses of 36 samples (13 species, two varieties and two forms) using the plastid sequences *trnK*, *trnL-F*, and *atpB-rbcL*. A strict consensus of the 72 most parsimonious trees obtained, which has an identical topology to the maximum likelihood trees, showed that a clade of *D. smilacinum* and *D. viridescens* was sister to the rest of the genus. In a clade composed of *D. sessile*, *D. lutescens*, *D. nantouense*, *D. uniflorum*, *D. shimadae*, and *D. kawakamii*, the former three species formed a subclade, while *D. sessile* var. *sessile* f. *sessile* from Kyushu, f. *minus*, var. *micranthum*, and *D. lutescens* were further grouped together. *Disporum sessile* var. *sessile* f. *sessile* from regions other than Kyushu, as well as *D. nantouense*, was excluded from this group. As *D. lutescens* differs greatly from *D. sessile* morphologically, they cannot be considered to be conspecific. Therefore, from a phylogenetic standpoint, two different species are possibly included under the name of *D. sessile* var. *sessile* f. *sessile*: one from Kyushu and the other from elsewhere. *Disporum nantouense* is possibly a variety of *D. sessile*. *Disporum sessile* var. *micranthum* Hatus., nom. nud. was validated.

Key words: Colchicaceae, *Disporum*, *Disporum lutescens*, *Disporum nantouense*, *Disporum sessile* var. *micranthum*, *Disporum sessile* var. *sessile* f. *minus*, *Disporum sessile* var. *shimadae* f. *intermedium*, Liliales, molecular phylogeny, taxonomy

*Disporum* Salisb. ex D. Don (Liliales-Colchicaceae) is composed of about 20 species that grow on the floor of deciduous/evergreen forests and in grasslands in the Himalaya, India, Southeast Asia, and East Asia (Hara 1988, Liang & Tamura 2000). *Disporum* is considered to be sister to the North American *Uvularia* L. (Tamura *et al.* 2004, Nguyen *et al.* 2013, Petersen *et al.* 2013), but *Disporum* differs from *Uvularia* (with capsules) in having berries (Nordenstam 1998).

Hara (1988), in his monograph of *Disporum*, proposed the following eight groups (A–H) of related species based on morphology: (A) *D. viridescens* (Maxim.) Nakai, *D. smilacinum* A. Gray, and *D. lutescens* (Maxim.) Koidz.; (B) *D. leschenaultianum* D. Don, *D. bodinieri* (H. Lév. & Vaniot) F. T. Wang & T. Tang, *D. leucanthum* H. Hara, and *D. tonkinense* T. Koyama; (C) *D. longistylum* (H. Lév. & Vaniot) H. Hara, *D. megalanthum* F. T. Wang & T. Tang, *D. trabeculatum* Gagnep., *D. hainanense* Merr., and *D. austrosinense* H. Hara; (D) *D. acuminatum* C. H. Wright; (E) *D. ovale* Ohwi; (F) American species; (G) *D. cantoniense* (Lour.) Merr. and *D. calcaratum* D. Don; (H) *D. uniflorum* Baker ex S. Moore and *D. sessile* D. Don ex Schult. Hara (1988) further hypothesized evolutionary relationships among the groups: he considered group A to be ancestral, groups B–F to be independently derived from group A, and groups G and H to be independently evolved from group C. Group E (*D. ovale*), however, is now considered to belong to *Streptopus* Michx. (Liliaceae) (Wang & Tang 1978, Fuse *et al.* 2001), and group F (which includes the American species) is included in Liliaceae as the independent genus, *Prosartes* D. Don (Shinwari *et al.* 1994a, c).

Tamura *et al.* (1992) reported chromosome numbers and karyotypes of 15 taxa (12 species, two varieties, and one form) of *Disporum*, in addition to four species of *Prosartes*. Results of the karyological study have raised questions about Hara's (1988) circumscription and grouping of the species of *Disporum*. First, although Hara (1988) considered *D. kawakamii* Hayata to be conspecific with *D. cantoniense*, they were shown to have different karyotypes and may be considered to be different species, as originally described. Second, Hara (1988) also considered *D. shimadae* Hayata to be conspecific with *D. sessile*, but they have different chromosome numbers. *Disporum shimadae* may be considered to be distinct from *D. sessile*. Third, *D. lutescens* has the same karyotype as *D. sessile* and *D. nantouense* S. S. Ying (=*D. taipingense* M. N. Tamura & Kawano), which differs from the karyotypes of the remaining members of group A (i.e., *D. viridescens* and *D. smilacinum*). The coherence of *D. sessile*, *D. lutescens,* and *D. nantouense*, and the separation of *D. lutescens* the group of from *D. viridescens* and *D. smilacinum* were novel findings by Tamura *et al.* (1992); the relationships should have been further tested using different methods.

A molecular phylogenetic analysis of *Disporum* using restriction fragment length polymorphisms (RFLPs) (Shinwari *et al.* 1994b) produced a strict consensus of the 24 equally most parsimonious trees of 11 species of *Disporum* that formed two clades, one consisting of *D. kawakamii* and *D. multiflorum* (Blume) D. Don, and the other consisting of *D. sessile*, *D. nantouense*, and *D. lutescens*, although bootstrap analysis was not performed. The remaining six species (*D. cantoniense* var. *sikkimense*, *D. leucanthum*, *D. shimadae*, *D. smilacinum*, *D. uniflorum*, *D. viridescens*) were excluded from the two clades. The latter clade corresponded to the aforementioned group with the same karyotype reported by Tamura *et al.* (1992). The fifty percent majority rule consensus of the most parsimonious trees did not necessarily agree with previous findings (e.g., *D. smilacinum* did not form a clade with *D. viridescens*, but did with *D. cantoniense* var. *sikkimense* H. Hara). The results obtained by RFLPs should be confirmed by molecular phylogenetic studies using DNA sequences.

We constructed the first molecular phylogenetic tree of *Disporum* based on DNA sequence data. Towards a better understanding of *Disporum* taxonomy, we analyzed the DNA sequences of the plastids *trnK*, *trnL–F*, and *atpB–rbcL* (4084 bp in total). The resulting tree revealed the evolutionary lineages of the species of *Disporum* and their relationships within the genus.

Among the taxa investigated was the *nomen nudum*, *Disporum sessile* var. *micranthum* Hatus., from the Ryukyus, Japan (Hatusima 1971). Although not formally published, the name var. *micranthum*, has been used in the Japanese flora (Satake 1982). Two of us (Tamura and Hotta) also considered var. *micranthum* to be distinguishable from other varieties of *D. sessile* and has provided it with an English description and a Latin diagnosis (see Appendix).

## Materials and Methods

### *Plant materials for molecular phylogenetic analysis*

DNA sequences of the plastid regions of *trnK* gene (including *matK*), *trnL–F* intergenic spacer and *atpB–rbcL* intergenic spacer (plus partial *atpB* gene) for 13 species, two varieties and two forms (36 OTUs) of *Disporum*, and two species of *Uvularia* (as the outgroup) were determined (Table 1). All of the sequence data are deposited in the DNA Data Bank of Japan (DDBJ) database. Voucher specimens are deposited in the herbaria of Kyoto University (KYO) and the Botanical Gardens, Graduate School of Science, Osaka City University.

TABLE 1. Sources of materials.

| Taxon | Locality | Voucher | Acc. no. (*trnK*) | Acc. no. (*trnL–F*) | Acc. no. (*atpB–rbcL*) |
|---|---|---|---|---|---|
| *Disporum austrosinense* H. Hara | Vietnam: Hanoi | *M. Ogisu s.n.* | AB858230 | AB858268 | AB858306 |
| *D. bodinieri* (H. Lév. & Vaniot) F. T. Wang & T. Tang | China: Yunnan, Mt. Yulongxueshan | *M. N. Tamura 4741* | AB858231 | AB858269 | AB858307 |
| *D. cantoniense* (Lour.) Merr. | China: unknown | *S. Fuse s.n.* | AB858232 | AB858270 | AB858308 |
| *D. kawakamii* Hayata | Taiwan: Yilan, Datong | *M. N. Tamura, S. Fuse, M. T. Kao & M. Tamura 10057* | AB858233 | AB858271 | AB858309 |
| *D. longistylum* (H. Lév. & Vaniot) H. Hara | China: Sichuan, Emeishan City | *M. N. Tamura, F. D. Pu & M. Tamura 7090* | AB858234 | AB858272 | AB858310 |
| *D. lutescens* (Maxim.) Koidz. | Japan: Wakayama Pref., Mt. Kohya | *M. Yamada 19* | AB858235 | AB858273 | AB858311 |
| *D. multiflorum* (Blume) D. Don | Indonesia: Sumatra, Mt. Tujuh | *M. Kato, H. Okada, N. Takamoto, M. Sugiura, Y. Mori & A. Takano 8413* | AB858236 | AB858274 | AB858312 |
| *D. nantouense* S. S. Ying | Taiwan: Yilan, Mt. Taiping | *M. N. Tamura, S. Fuse, M. T. Kao & M. Tamura 10051* | AB858237 | AB858275 | AB858313 |
| *D. sessile* D. Don ex Schult. | | | | | |
| var. *sessile* f. *sessile* | Japan: Aomori Pref., Nishimeya | *M. N. Tamura & S. Fuse 10093* | AB858238 | AB858276 | AB858314 |
| | Japan: Akita Pref., Oga | *M. N. Tamura & S. Fuse 10139* | AB858239 | AB858277 | AB858315 |
| | Japan: Shizuoka Pref., Atami | *M. N. Tamura & S. Fuse 10265* | AB858240 | AB858278 | AB858316 |
| | Japan: Fukui Pref., Mt. Kanmuri | *M. N. Tamura, M. Nagaoka & A. Yokokawa 10262* | AB858241 | AB858279 | AB858317 |
| | Japan: Shiga Pref., Mt. Ibuki | *M. N. Tamura 7097* | AB858242 | AB858280 | AB858318 |
| | Japan: Nara Pref., Gose | *M. Yamada 1* | AB858243 | AB858281 | AB858319 |
| | Japan: Tokushima Pref., Yamakawa | *M. N. Tamura & S. Fuse 10047* | AB858244 | AB858282 | AB858320 |
| | Japan: Fukuoka Pref., Mts. Sefuri | *M. Yamada 68* | AB858245 | AB858283 | AB858321 |
| | Japan: Kumamoto Pref., Mt. Kinbou | *M. N. Tamura & S. Fuse 9003* | AB858246 | AB858284 | AB858322 |
| | Japan: Kagoshima Pref., Makizono | *M. Hotta s.n.* | AB858247 | AB858285 | AB858323 |
| | Korea: Gyeongsangbuk-do, Ulleungdo Isl. | *M. N. Tamura, J. H. Pak & M. Tamura s.n.* | AB858248 | AB858286 | AB858324 |
| var. *sessile* f. *macrophyllum* (Koidz.) H. Hara | Japan: Akita Pref., Choukai-Kohgen | *M. N. Tamura & S. Fuse 10125* | AB858249 | AB858287 | AB858325 |
| var. *sessile* f. *minus* (Miq.) H. Hara | Japan: Wakayama Pref., Mt. Kohya | *M. Yamada 11* | AB858250 | AB858288 | AB858326 |
| | Japan: Wakayama Pref., Ohtou | *M. N. Tamura, J. Yamashita & T. Inada s.n.* | AB858251 | AB858289 | AB858327 |
| | Japan: Kagoshima Pref., Kirishima | *M. N. Tamura & S. Fuse 9025* | AB858252 | AB858290 | AB858328 |
| | Japan: Kagoshima Pref., Mt. Shibi | *M. Hotta s.n.* | AB858253 | AB858291 | AB858329 |
| | Japan: Kagoshima Pref., Mt. Inao | *M. Hotta s.n.* | AB858254 | AB858292 | AB858330 |
| var. *micranthum* Hatus. ex M.N. Tamura & M. Hotta | Japan: Kagoshima Pref., Amami-Ohshima Isl. | *M. Hotta s.n.* | AB858255 | AB858293 | AB858331 |
| | Japan: Kagoshima Pref., Tokunoshima Isl. | *M. Hotta s.n.* | AB858256 | AB858294 | AB858332 |
| *D. shimadae* Hayata | Taiwan: Keelung | *M. Tamura & M. T. Kao s.n.* | AB858257 | AB858295 | AB858333 |
| *D. smilacinum A.Gray* | | | | | |
| var. *smilacinum* | Japan: Nara Pref., Kashiba | *S. Fuse s.n.* | AB858258 | AB858296 | AB858334 |
| | Japan: Hiroshima Pref., Hatsukaichi | *H. Okada s.n.* | AB858259 | AB858297 | AB858335 |
| | Korea: Gangwon-do, Mt. Odae | *M. N. Tamura, N. S. Lee, S. H. Yeau & M. Tamura 10064* | AB858260 | AB858298 | AB858336 |
| | Korea: Gyeonggi-do, Mt. Chunma | *M. N. Tamura, N. S. Lee & S. H. Yeau 15402* | AB858261 | AB858299 | AB858337 |
| | Korea: Chungcheongnam-do, Anmyeondo Isl. | *M. N. Tamura, N. S. Lee & S. H. Yeau 10082* | AB858262 | AB858300 | AB858338 |
| var. *ramosum* Nakai | Korea: Gyeonggi-do, Mt. Chunma | *M. N. Tamura, N. S. Lee & S. H. Yeau 15403* | AB858263 | AB858301 | AB858339 |
| *D. uniflorum* Baker ex S. Moore | Korea: Gyeonggi-do, Gwangneung | *M. N. Tamura, N. S. Lee, S. H. Yeau & M. Tamura 10070* | AB858264 | AB858302 | AB858340 |
| *D. viridescens* (Maxim.) Nakai | Korea: Gyeonggi-do, Gwangneung | *M. N. Tamura, N. S. Lee, S. H. Yeau & M. Tamura 10073* | AB858265 | AB858303 | AB858341 |
| *Uvularia perfoliata* L. | U.S.A.: eastern | *S. Kawano, H. Takasu, A. Hiratsuka & M. N. Tamura s.n.* | AB858266 | AB858304 | AB858342 |
| *U. sessilifolia* L. | U.S.A.: eastern | *S. Kawano, H. Takasu, A. Hiratsuka & M. N. Tamura s.n.* | AB858267 | AB858305 | AB858343 |

TABLE 2. Name, direction, nucleotide sequence and source of primers used in the present study. The primers with an asterisk were used for both PCR amplification and DNA sequencing, the others only for DNA sequencing.

| Primer name | Direction | Sequence (5' – 3') | Source |
|---|---|---|---|
| [*trnK* (including *matK*)] | | | |
| *trnK*-11* | sense | CTCAACGGGTAGAGTACTCG | Liston & Kadereit (1995) |
| *trnK*-601F* | sense | AGAGTGATCCGGTTGCAA | Newly designed |
| *matK*-1105F | sense | GATCCATTGGGCGCAACCA | Newly designed |
| *matK*-1660F | sense | GAAATGGAAATGTTACCTTG | Newly designed |
| *matK*-8F | sense | TCGACTTTCTTGTGCTAGAACTTT | Steele & Vilgalys (1994) |
| *trnK*-412R | antisense | CCTTTCCCGAATCAGGCACTAA | Newly designed |
| *matK*-887R | antisense | GACCATGAGCAAGCGTATA | Newly designed |
| *matK*-1876R | antisense | ACACTTGAAAGATATCCCAA | Newly designed |
| *matK*-8R* | antisense | AAAGTTCTAGCACAAGAAAGTCGA | Newly designed |
| *trnK*-2621* | antisense | AACTAGTCGGATGGAGTAG | Liston & Kadereit (1995) |
| [*trnL–F*] | | | |
| *trnL*-e* | sense | GGTTCAAGTCCCTCTATCCC | Taberlet *et al.* (1991) |
| *trnF*-f* | antisense | ATTTGAACTGGTGACACGAG | Taberlet *et al.* (1991) |
| [*atpB–rbcL*] | | | |
| *rbcL*-NF* | sense | TAGTTTCTGTTTGTGGTGACAT | Terachi (1993) |
| *rbcL*-TF | sense | CCTCCCTACAACTCATGAA | Newly designed |
| *rbcL*-MF | sense | CTAAAATGGAGTTCGGGTT | Newly designed |
| *atpB*-MR | antisense | ATTCGAACCCGAACTCCA | Newly designed |
| *atpB*-NR'* | antisense | AAGTAGGATTGGTTCTCAT | Modified Terachi (1993) |
| *atpB*-TR* | antisense | CTCATAGCGACAGTTCTAAC | Newly designed |

### *DNA extraction, PCR amplification and DNA sequencing*

Total genomic DNA was extracted from fresh leaves by the slightly modified cetyltrimethylammonium bromide (CTAB) method of Stewart & Via (1993). The regions of *trnK*, *trnL–F* and *atpB–rbcL* were amplified using the polymerase chain reaction (PCR) with a programmable temperature-control system PC-700 (Astec) or GeneAmp PCR System 9600 (Applied Biosystems). The amplification reaction mixture was prepared using TaKaRa Ex Taq DNA polymerase following its instruction manual (Takara Shuzo). We used four, two and three PCR primers (Table 2) for the amplification of *trnK*, *trnL–F* and *atpB–rbcL* regions, respectively. This was followed by a 37 cycle denaturation at 94°C for 40 sec, annealing at 56°C for 40 sec, and extension at 72°C for 2.5 min, in addition to an initial denaturation at 94°C for 3 min and a final extension at 72°C for 5 min. We purified the PCR products with MicroSpin S-300 HR Columns (Pharmacia Biotech).

Direct sequencing was conducted with ten, two and six primers (Table 2) for *trnK*, *trnL–F* and *atpB–rbcL* regions, respectively, using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's instruction. We manually aligned the DNA sequences obtained.

### *Phylogenetic analysis*

We employed the methods of maximum parsimony (MP) and maximum likelihood (ML) for phylogeny reconstruction using PAUP* version 4.0 beta 10 (Swofford 2002).

In the MP analysis, heuristic search option of random addition sequence (10000 replications), the tree bisection and reconnection (TBR) swapping algorithm and multiple parsimonious trees

(MULPARS) were used to find the most parsimonious trees. Only the base substitutions were treated as characters for phylogenetic reconstruction. However, the polyA (only adenine linked continuously) regions of position 142–152, 2800–2809 and 3809–3824 (counted along the aligned DNA sequences from the first nucleotide of our combined sequence data), and the polyT (only thymine linked continuously) regions of position 477–492 and 3004–3019 were excluded from the phylogenetic analyses due to difficulties in identifying homologous bases. All base substitutions were equally weighted. The gaps were treated as missing values. The consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated with PAUP*, including uninformative characters. Bootstrap analysis with 1000 replications (heuristic; 1000 random; TBR) was performed using PAUP*.

In the ML analysis, the best model of nucleotide evolution was estimated using Modeltest 3.7 (Posada & Crandall 1998). The models selected by the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC) were K81uf+G and K81uf+I, respectively. Both models were implemented (heuristic; 100 random; TBR), according to the parameters specified by the models. ML bootstrap analysis with 100 replications (heuristic; 10 random; TBR) was performed using PAUP*.

Congruence among the data sets of the three plastid regions, *trnK*, *trnL–F* and *atpB–rbcL*, was examined with the incongruence length difference (ILD) test (Farris *et al.* 1994), implemented in PAUP*, as the partition homogeneity test. The tests were performed with 100 replications (heuristic; 100 random; TBR), and MULPARS.

### *Validation of* Disporum sessile *var.* micranthum

To characterize *Disporum sessile* var. *micranthum*, which had not been formally published, herbarium specimens at KAG, KYO and TI were examined.

## Results

### *DNA sequence variation within* Disporum

The *trnK* gene ranged from 2650 to 2659 bp, the *trnL–F* intergenic spacer from 422 to 438 bp, and the *atpB–rbcL* intergenic spacer (plus partial *atpB* gene) from 973 to 989 bp. Values of pairwise sequence divergence ranged from 0.0 to 0.9% for *trnK*, to 3.2% for *trnL–F* and to 1.2% for *atpB–rbcL*.

### *Phylogenetic analysis of* Disporum *and* Uvularia *(the latter as an outgroup)*

Of the 2668 bp of *trnK* sequences that were aligned for analysis (excluding the 11-base polyA and 16-base polyT regions), 82 bp (3.1%) were variable, and 37 bp (1.4%) were phylogenetically informative. Of the 432 bp of aligned *trnL–F* sequences (excluding the 10-base polyA and 16-base polyT regions), 28 bp (6.5%) were variable, and 12 bp (2.8%) phylogenetically informative. Of the 984 bp of aligned *atpB–rbcL* sequences (excluding the 16-base polyA region), 28 bp (2.8%) were variable, and 12 bp (1.2%) phylogenetically informative. The partition-homogeneity test among the data sets of three plastid regions, *trnK*, *trnL–F* and *atpB–rbcL*, found a P value of 1.00. Considering an incongruence threshold of 0.05, we concluded that the data sets of three plastid regions were combinable. The combined analysis of plastid *trnK*, *trnL–F* and *atpB–rbcL* sequences resulted in 72 equally most parsimonious trees with 147 steps. The CI is 0.96, the RI 0.97, and the RC 0.94.

The topology of the MP strict consensus tree was identical to the topology of the ML tree using the models selected by both hLRT and AIC (Fig. 1). According to the MP strict consensus tree (Fig. 1), *Disporum* formed a monophyletic group with 100% bootstrap support. In *Disporum*, two major clades were formed: one (with 93% bootstrap support) consisting of the species with widely opening or infundibular flowers (i.e., *D. smilacinum* and *D. viridescens*), and the other (73%) consisting of the 11 remaining species with tubular, campanulate, or obconical flowers. The

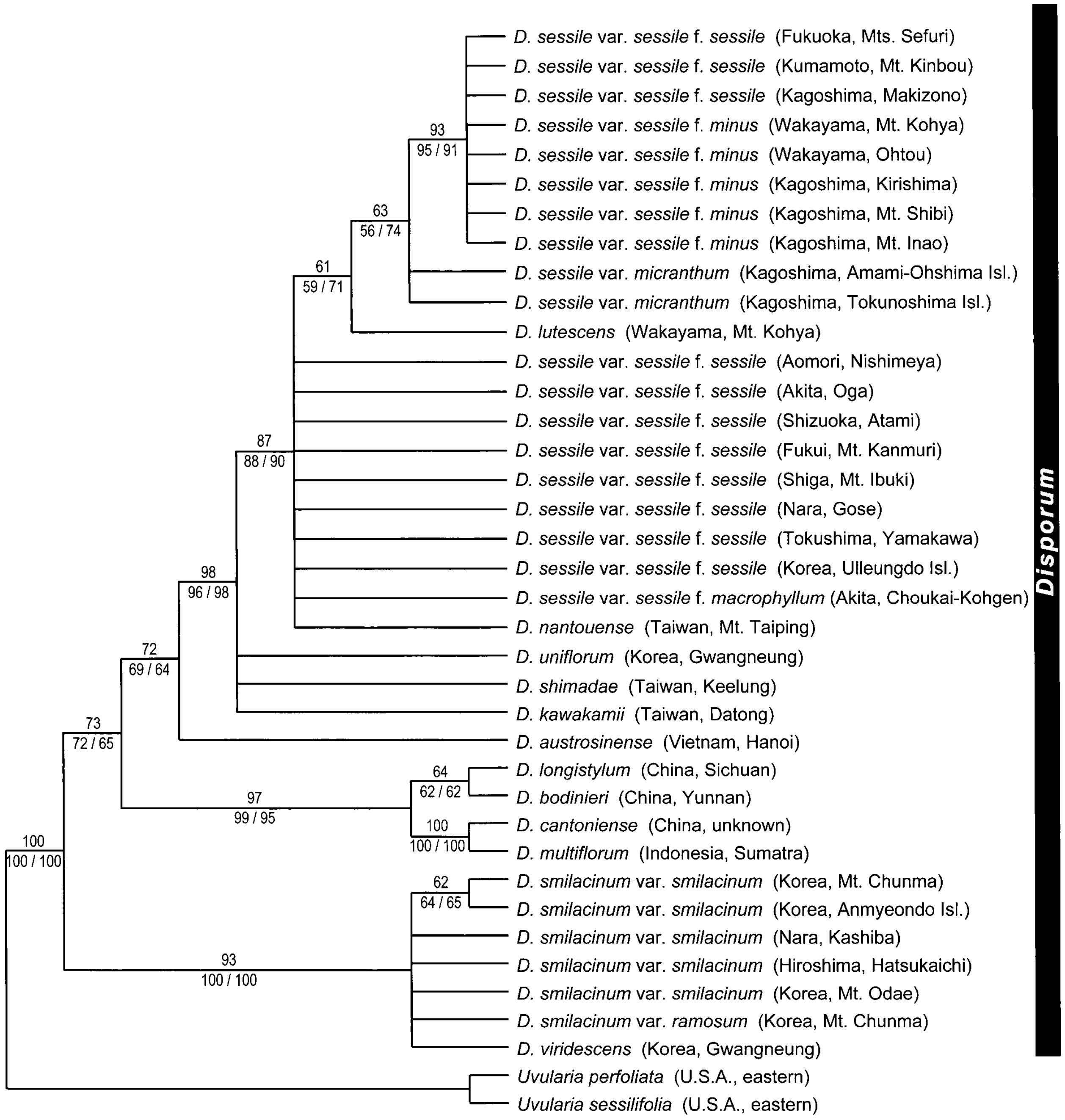
D. sessile var. sessile f. sessile (Fukuoka, Mts. Sefuri)
D. sessile var. sessile f. sessile (Kumamoto, Mt. Kinbou)
D. sessile var. sessile f. sessile (Kagoshima, Makizono)
D. sessile var. sessile f. minus (Wakayama, Mt. Kohya)
D. sessile var. sessile f. minus (Wakayama, Ohtou)
D. sessile var. sessile f. minus (Kagoshima, Kirishima)
D. sessile var. sessile f. minus (Kagoshima, Mt. Shibi)
D. sessile var. sessile f. minus (Kagoshima, Mt. Inao)
D. sessile var. micranthum (Kagoshima, Amami-Ohshima Isl.)
D. sessile var. micranthum (Kagoshima, Tokunoshima Isl.)
D. lutescens (Wakayama, Mt. Kohya)
D. sessile var. sessile f. sessile (Aomori, Nishimeya)
D. sessile var. sessile f. sessile (Akita, Oga)
D. sessile var. sessile f. sessile (Shizuoka, Atami)
D. sessile var. sessile f. sessile (Fukui, Mt. Kanmuri)
D. sessile var. sessile f. sessile (Shiga, Mt. Ibuki)
D. sessile var. sessile f. sessile (Nara, Gose)
D. sessile var. sessile f. sessile (Tokushima, Yamakawa)
D. sessile var. sessile f. sessile (Korea, Ulleungdo Isl.)
D. sessile var. sessile f. macrophyllum (Akita, Choukai-Kohgen)
D. nantouense (Taiwan, Mt. Taiping)
D. uniflorum (Korea, Gwangneung)
D. shimadae (Taiwan, Keelung)
D. kawakamii (Taiwan, Datong)
D. austrosinense (Vietnam, Hanoi)
D. longistylum (China, Sichuan)
D. bodinieri (China, Yunnan)
D. cantoniense (China, unknown)
D. multiflorum (Indonesia, Sumatra)
D. smilacinum var. smilacinum (Korea, Mt. Chunma)
D. smilacinum var. smilacinum (Korea, Anmyeondo Isl.)
D. smilacinum var. smilacinum (Nara, Kashiba)
D. smilacinum var. smilacinum (Hiroshima, Hatsukaichi)
D. smilacinum var. smilacinum (Korea, Mt. Odae)
D. smilacinum var. ramosum (Korea, Mt. Chunma)
D. viridescens (Korea, Gwangneung)
Uvularia perfoliata (U.S.A., eastern)
Uvularia sessilifolia (U.S.A., eastern)
Disporum
93
95 / 91
63
56 / 74
61
59 / 71
87
88 / 90
98
96 / 98
72
69 / 64
73
72 / 65
100
100 / 100
64
62 / 62
97
99 / 95
100
100 / 100
62
64 / 65
93
100 / 100

Fig. 1. Strict consensus of 72 equally most parsimonious trees resulting from the combined maximum parsimony (MP) analysis of plastid *trnK*, *trnL–F* and *atpB–rbcL* DNA sequence data of *Disporum* and *Uvularia* (the latter as an outgroup). The topology of the maximum likelihood (ML) tree using either of the models, i.e. the model selected by hierarchical likelihood ratio test (hLRT) and the model selected by Akaike information criterion (AIC), is identical to the topology of this figure. Numbers above branches indicate MP bootstrap values (percentages of 1000 replicates). Numbers below branches indicate ML (hLRT) bootstrap values/ML (AIC) bootstrap values (percentages of 100 replicates).

latter clade was composed of two clades: one (97%) consisting of the four species from Southeast Asia and the inland regions of China (*D. bodinieri*, *D. longistylum*, *D. cantoniense*, and *D. multiflorum*) and the other (72%) consisting of the seven species distributed in coastal regions from Vietnam to eastern Asia. *Disporum cantoniense* and *D. multiflorum* formed a common clade (100%). In the clade of species from coastal regions of Vietnam to eastern Asia, the species from regions north of Taiwan further formed a clade (98%). *Disporum austrosinense* from Vietnam to Guangdong, China, was excluded from the clade. In this clade, *D. sessile*, *D. lutescens*, and *D. nantouense* further formed a clade (87%), and *D. uniflorum*, *D. shimadae*, and *D. kawakamii* were excluded. In the latter clade, *D. sessile* var. *sessile* f. *sessile* from Kyushu, f. *minus*, var. *micranthum*, and *D. lutescens* further formed a clade (61%), and *D. sessile* var. *sessile* f. *sessile* from regions other than Kyushu, f. *macrophyllum*, and *D. nantouense* were excluded. In the last clade, *D. lutescens* was separated from the remaining plants (63%), among which *D. sessile* var. *sessile* f. *sessile* from Kyushu and f. *minus* were grouped (93%), and the samples of *D. sessile* var. *micranthum* were excluded from the group.

## Discussion

Hara (1988) regarded the obconical flowers of *Disporum lutescens* as being similar in shape to the widely opening or infundibular flowers of *D. smilacinum* and *D. viridescens*, and considered the three species to be related. In contrast, Tamura *et al.* (1992) considered *D. lutescens* to be related to *D. sessile* and *D. nantouense* based on the similarity of their karyotypes. The molecular phylogenetic results by both RFLPs (Shinwari *et al.* 1994b) and DNA sequencing (this study) supported the opinion of Tamura *et al.* (1992), and revealed that *D. lutescens* is not at all related to *D. smilacinum* and *D. viridescens*.

In *Disporum sessile* var. *sessile* f. *sessile,* the plants from Kyushu (Fukuoka, Kumamoto and Kagoshima) were shown to be phylogenetically distinct from plants from the other regions and were grouped with *D. sessile* var. *sessile* f. *minus*, var. *micranthum*, and *D. lutescens*. As *D. lutescens* differs greatly from *D. sessile* morphologically, they cannot be considered to be conspecific. Therefore, from a phylogenetic standpoint, *D. sessile* var. *sessile* f. *sessile* from Kyushu should be considered as a species distinct from *D. sessile* var. *sessile* f. *sessile* from other regions. The bootstrap value linking *D. sessile* var. *sessile* f. *sessile* from Kyushu to *D. lutescens* was low (61%), however, and clear morphological gaps between *D. sessile* var. *sessile* f. *sessile* from Kyushu and from individuals from other regions have not yet been detected. We await future detailed morphological and molecular studies before proposing *D. sessile* var. *sessile* f. *sessile* from Kyushu and *D. sessile* var. *sessile* f. *sessile* from other regions as separate species.

*Disporum sessile* var. *sessile* f. *minus* formed a robust clade (93%) with *D. sessile* var. *sessile* f. *sessile* from Kyushu. Although delimitation of the two forms is ambiguous, typical individuals of the two forms differ in size, ramifications, flower number, and filament surface (pers. obs.). Further morphological and molecular studies are needed to determine if these two forms are indeed different taxa. The two samples of *D. sessile* var. *micranthum* did not form a single clade, possibly because of insufficient sequence data. Both samples, however, were excluded from the clade consisting of f. *sessile* from Kyushu and f. *minus*. The results do not contradict the recognition of var. *micranthum*. Additional molecular data are needed to determine whether var. *micranthum* represents a single lineage.

Hara (1988) considered *Disporum nantouense* (=*D. sessile* var. *shimadae* f. *intermedium* H. Hara) to be related to *D. shimadae* from a morphological standpoint while Liang & Tamura (2000) reported the relationship of *D. sessile* and *D. nantouense* from both morphological and karyological (Tamura *et al.* 1992) standpoints. The molecular phylogenetic results by both RFLPs (Shinwari *et al.* 1994b) and DNA sequencing in this study support the latter relationship because *D. nantouense* was included in the clade of *D. sessile* plus *D. lutescens*. Although *D. nantouense* is generally similar to *D. sessile* in morphology, they differ minutely in leaf shape, flower color, and degree of connation of styluli. Thus, they merit recognition as different taxa, with *D. nantouense* possibly a variety of *D. sessile*. Further morphological and molecular studies with sufficient samples of *D. nantouense*, *D. sessile*, and *D. lutescens* are needed to determine the exact taxonomic position of *D. nantouense*.

Hara (1988) treated *Disporum shimadae* and *D. kawakamii* as varieties of *D. sessile* and *D. cantoniense*, respectively. Tamura *et al.* (1992)

disagreed with these groupings, and insisted that *D. shimadae* and *D. kawakamii* should be treated as different species from *D. sessile* and *D. cantoniense*, respectively, because *D. sessile* has a chromosome number based on $x$=8 while the karyotype of *D. shimadae* is based on $x$=7. Additionally, Tamura *et al.* (1992) noted that *D. cantoniense* and *D. kawakamii* have different karyotypes. Results from our molecular phylogenetic study excluded *D. shimadae* and *D. kawakamii* from the clades of *D. sessile* and *D. cantoniense*, respectively, and thus support the findings of Tamura *et al.* (1992). Hara (1988) treated *D. multiflorum* as a variety of *D. cantoniense*. Our study revealed that *D. multiflorum* and *D. cantoniense* form a robust clade (100%), supporting a close relationship between them, as Hara (1988) suspected.

Among the extant species of *Disporum*, Hara (1988) considered *D. smilacinum*, *D. viridescens* and *D. lutescens* with more or less widely opening flowers to be ancestral. *Uvularia*, however, which is sister to *Disporum* (Tamura *et al.* 2004, Nguyen *et al.* 2013, Petersen *et al.* 2013), as well as the species of *Disporum* other than *D. smilacinum*, *D. viridescens* and *D. lutescens*, have narrower (tubular, campanulate or obconical) flowers. This study, together with Tamura *et al.* (2004), Nguyen *et al.* (2013) and Petersen *et al.* (2013), raised the possibility that widely opening flowers are not necessarily ancestral in *Disporum*, but derived from narrower flowers in the lineage of *D. smilacinum* and *D. viridescens*. Further studies are needed to determine the ancestral shape of the flowers of *Disporum*. We agree with Hara (1988) that *D. smilacinum* and *D. viridescens* are distantly related to *D. sessile*.

Although we intended to discuss Hara's (1988) grouping of the species of *Disporum* and the evolutionary relationships among the groups, the lack of sufficient samples from China and Southeast Asia have postponed this subject for a future study. Further, we were unable to address some of the issues raised by Tamura *et al.* (1992). For example, *D. cantoniense/D. calcaratum* contain individuals with a base chromosome number of both $x$=7 and $x$=8. Further morphological and molecular studies using more samples from China and Southeast Asia are needed to confirm the present broad circumscription of *D. cantoniense/ D. calcaratum*.

## Appendix

**Disporum sessile** D. Don ex Schult. var. **micranthum** Hatus. ex M. N. Tamura & M. Hotta, **var. nov.** —Fig. 2.

*Disporum sessile* D. Don ex Schult. var. *micranthum* Hatus., Fl. Ryukyus: 787 (1971); Satake in Satake *et al.*, Wild Fl. Jap. Herb. Pl. 1: 48 (1982); M. Hotta in Bull. Kagoshima Pref. College 55: 19, 89 (2004), nom. nud.

Affine *Disporo sessili* var. *sessili* est, sed floribus minoribus et odoratis differt.

*Typus*. Japan, Kagoshima Pref., Amami-Ohshima Isl., early March 1924 (fl.), Z. *Tashiro s.n.* (holo-TI, iso-KYO).

Stolons terrestrial, creeping. Winter buds for next aerial stems from both rhizome and stolons. Flowers fragrant. Tepals 1.5–2.2 cm long, white, only slightly greenish distally. Stamens 6; filaments 0.8–1.2 cm long, sparsely papillose proximally; anthers 3–4 mm long.

*Japanese name*. Nangoku-houchaku-so (Hatusima 1971).

*Distribution and habitat*. Kuchinoerabu Isl., Tokara Islands and Amami Islands (north of Tokunoshima Isl.), Kagoshima Pref., Japan. In forests and bamboo thickets, 100-700 m alt.

*Other specimens examined*. Japan, Kagoshima Pref.: Kuchinoerabu Isl., *Hatusima 13506* (KAG). —Kuchinoshima Isl., 4 May 1988, *Maeda s.n.* (KAG). —Nakanoshima Isl., *Naiki 5258* (KYO); 31 Mar. 1978, *Nakayama s.n.* (KAG); *Sako & Kawanabe 1727* (KAG), *1921* (KAG, KYO). —Gaja Isl., *Sako & Kawanabe 2271* (KAG). —Suwanose Isl., 11–15 Dec. 1971, *Nakayama s.n.* (KAG); 29 Mar. 1977, *Tamari, Miyata & Hatanaka s.n.* (KAG). —Akuseki Isl., *Hatusima 16045* (KAG); 10 Aug. 1933, *Naito s.n.* (KAG); *Sako 6245* (KAG, KYO, TI). —Amami-Ohshima Isl., *Ferrié 135* (KYO); *Fukuoka 7965* (KYO); *Hatusima & Sako 21809* (TI), *21863* (KYO, TI); 1 Apr. 1937, *Iwamasa s.n.* (TI); *Kobayashi 1246* (TI); 30 Apr. 1923, 5 May 1923, *Koidzumi s.n.* (KYO); *Murata 56324* (KYO); *Naiki 5527* (KYO); *Ohba 863124* (TI); *Sako 5331* (KYO); *Setoguchi JP2608* (KYO); *Tamura & Kakezawa 20496*, *20498*, *20500*, *20503*, *20504*, *20506*

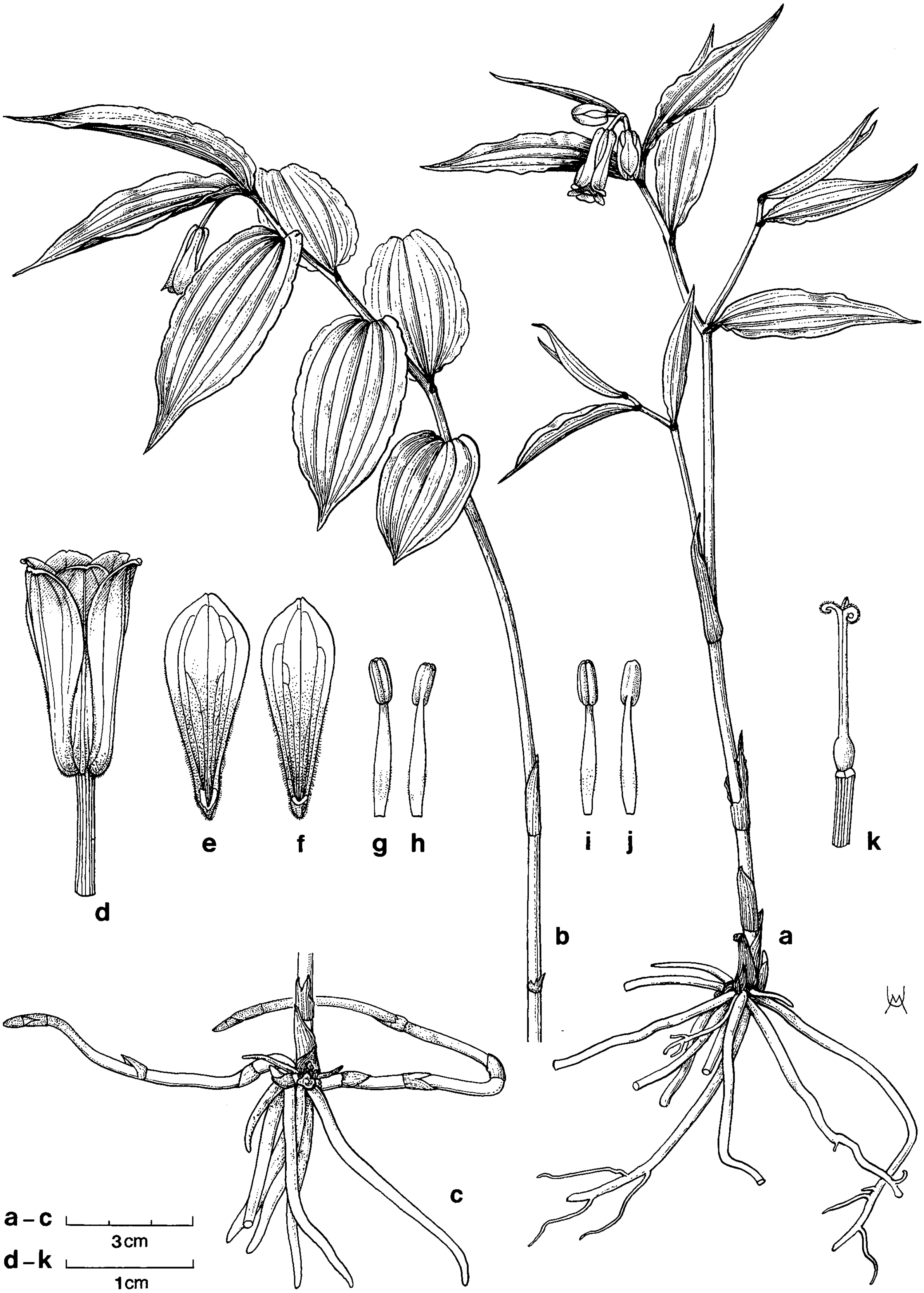

Fig. 2. *Disporum sessile* D. Don ex Schult. var. *micranthum* Hatus. ex M. N. Tamura & M. Hotta. a, Habit of 3-flowered individual (*Tamura & Kakezawa 20504*, KYO). b, 1-flowered leafy shoot (*Tamura & Kakezawa 20500,* KYO). c, Underground portion of young-fruited individual with two terrestrial stolons (*Tamura & Kakezawa 20496,* KYO). d, Flower in lateral view; e, adaxial surface of outer tepal; f, adaxial surface of inner tepal; g, abaxial surface of outer stamen; h, adaxial surface of outer stamen; i, abaxial surface of inner stamen; j, adaxial surface of inner stamen; k, pistil in lateral view.

(KYO); *Tamura & Tamura 3178* (KYO); *Tamura, Yamashita & Kondo 11631* (KYO); *Tanaka 482* (TI); *Tsugaru 208* (KYO); 29 Jun. 1999, *Yamazaki s.n.* (TI); *Yasuda, Kokubugata & Saito 5319* (KYO). —Tokunoshima Isl., *Iwatsuki, Takasu, Mitsuta, Terao & Fukuoka 55, 568* (KYO); *Kato & Miki 244* (KYO); *Nagamasu 1728* (KYO); *Ohba 355* (KYO); *Ohba & Akiyama 8903027* (TI); *Tamura, Nakada, Nagamasu & Tamura 79* (KYO).

*Note. Disporum sessile* var. *sessile* has subterranean stolons from which winter buds are exclusively produced (Umebayashi & Shimizu 2000), larger odorless flowers with distally greenish, white tepals 2–3(–4) cm long, filaments (1.2–)1.5–2(–2.3) cm long and densely papillose proximally, and anthers, (3.5--)4–6 mm long.

## Conclusion

(1) *Disporum sessile* var. *micranthum* is morphologically distinguishable from *D. sessile* var. *sessile*, as validated in this study. Molecular phylogeny did not contradict this finding.

(2) *Disporum nantouense* should possibly be treated as a variety of *D. sessile*.

(3) *Disporum lutescens* is related to *D. sessile* (and *D. nantouense*) rather than to *D. smilacinum* and *D. viridescens*.

(4) *Disporum smilacinum* and *D. viridescens* are distantly related to *D. sessile*.

(5) *Disporum sessile* var. *sessile* f. *sessile* from Kyushu is possibly specifically distinct from *D. sessile* var. *sessile* f. *sessile* from regions other than Kyushu.

(6) *Disporum kawakamii* and *D. shimadae* should be treated as distinct species.

(7) The recognition of *Disporum cantoniense* var. *multiflorum* is supported.

(8) Widely opening flowers are not necessarily ancestral in *Disporum*.

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